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Studies carried out during the past year have confirmed and extended our novel discovery that the cell cycle control protein cyclin dependent kinase 6 (CDK6) can bind to the androgen receptor (AR) and markedly stimulate its transcriptional activity in human prostate cancer cells. Furthermore, this effect does not require the kinase activity of CDK6. In studies with variants of the AR we found that short CAG repeats in exon 1 of the AR, which occur more frequently in African American (AA) men with prostate cancer, were more responsive to the stimulatory effects of CDK6 than an AR with long CAG repeats. In addition, a point mutated AR (T877A), known to frequently occur in human prostate cancer, displayed dramatic stimulation by CDK6 in cells treated with dihydrotestosterone DHT, b-estradiol or progesterone. Taken together, these results suggest that CDK6 may play an important role in enhancing the development and progression of prostate cancer. It may therefore provide a prognostic marker and potential target for the prevention and therapy of prostate cancer.

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Introduction

Prostate cancer is currently the most frequently diagnosed cancer and the second leading cause of cancer deaths in men in the United States. In 1999 about 179,300 new cases were diagnosed and 37,000 men died from this disease (1). Furthermore, prostate cancer incidence is expected to double by the year 2030 (2). Despite the magnitude of this disease, the precise exogenous causative factors and the pathogenesis of this disease at the cellular and molecular levels are poorly understood. The Androgen Receptor (AR) plays a critical role in the development of prostate cancer, and abnormalities in the AR and/or in AR-mediated signaling pathways play an important role in the progression of this disease, and in responses to therapy. We have focused on proteins that regulate cell cycle progression because it is becoming increasingly apparent that abnormalities in the expression of these proteins often occurs during the multistage carcinogenic process (3). It is known that *in vivo* cyclin D1 binds to CDK4 or CDK6, thus activating their kinase activities, to enhance the G1 to S progression of the cell cycle progression (3). During the course of our studies on possible effects of these cell cycle control proteins on AR mediated functions we discovered an unexpected stimulatory effect of CDK6 itself. Our assay system utilized the AR negative PC3 human prostate cancer cell line for transient transfection reporter assays, employing either probasin or prostate specific antigen (PSA) promoter-luciferase reporter constructs (4), in which these promoters contain AR responsive elements (ARE). We found that co-transfection with a plasmid encoding a full-length CDK6 plus a plasmid encoding the AR markedly stimulated transcription from either of these reporters when the cells were also treated with dihydrotestosterone (20 nM DHT). However, similar co-transfection assays with CDKs 1, 2 or 4 did not stimulate this activity. The stimulatory effect required transfection with the AR, since PC3 cells do not express the AR, and the addition of

DHT to activate the AR. Using PSA promoter reporter assays, we found that CDK6 also markedly stimulated the transcriptional activity of the endogenous AR present in the LNCaP human prostate cancer cell line. Taken together, these results provided the basis for this project. Our overall goal is to establish the fact that CDK6 can bind to and activate the transcriptional activity of the AR to elucidate the mechanism of this effect, and to study the possible relevance of these findings to the development and progression of human prostate cancer.

Body:

Research accomplished on specific tasks during year two:

Statement of Work

Task 1. Analyze in detail the molecular mechanisms by which CDK6 markedly stimulates the activity of the androgen receptor (AR) in prostate cancer cells

a. Determine whether His-tagged CDK6 can physically associate with the AR, independent of cyclin D1.

In last year's progress report we presented evidence from co-immunoprecipitation experiments that in intact cells CDK6 can bind to the AR and that this does not require the presence of cyclin D1. During the past year we have extended these studies using a series of point mutants of CDK6. We used 293T cells since they express relatively low levels of endogenous cyclin D1 (5). The cells were co-transfected with the AR, CDK6 or a series of mutated constructs of CDK6. Co-immunoprecipitation studies of these cell extracts indicated that CDK6 binds to the AR protein *in vivo*. Furthermore, we found that point mutations in the INK binding (CDK6R31C), cyclin D1 binding (CDK6RE60,61AA) or ATP transfer(CDK6D146N) regions of CDK6 are not essential for binding to the AR (Fig. 1). In studies described in last year's Annual Report we found that none of these mutations interfered with the stimulatory effect of CDK6 on the AR in PSA-luciferase reporter assays in PC3 cells. Indeed, the results obtained with

HAK6R31C indicated that a mutation in CDK6 at residue 31, previously shown to relieve $p16^{INK4a}$ inhibition (6), caused a marked increase in activity. This finding suggests that the endogenous $p16^{INK4a}$ in PC3 cells exerts an inhibitory effect on CDK6 activation of the AR.

b. Determine the specific sequences in CDK6 that are responsible for it stimulatory effects of the AR.

In last year's Annual Report we described detailed studies on the effects of specific point mutations and deletions in CDK6 on its ability to stimulate the transcriptional activity of the AR. Our major conclusion was that the binding sites for $p16^{INK4a}$, the ATP binding site, and the cyclin D1 binding site in CDK6 are not required for this activity. The compound 3-ATA, a selective CDK4/6 – cyclin D1 kinase inhibitor (Alexis Corp. San Diego), did not inhibit CDK activation of the AR. These findings indicate that CDK6 does not stimulate the transcriptional activity of the AR by phosphorylating the AR. Therefore, our original Task 2 that addressed this question has now been completed.

c. Effects of decreasing cellular level of CDK6.

To extend our studies on CDK6, during the past year we examined the effects of the compound indole-3-carbinol (I3C), a known inhibitor of CDK6 expression (7). PC3 human prostate cancer cells that lack expression of the AR were-co-transfected with an androgen-responsive probasin luciferase reporter construct together with an AR expression plasmid and a plasmid that encodes CDK6. The cells were then grown in the presence of 20 nM DHT and treated with 200 or 500 μM 14C for 24 or 48 hours. We found that I3C caused a significant decrease in cellular levels of the endogenous CDK6 protein (Fig. 2, left panel), and that this was associated with a marked decrease in DHT stimulation of PSA luciferase activity (Fig. 2; right panel). These results also suggest that the basal PSA luciferase reporter activity observed in PC3 cells not transfected with exogenous CDK6 is due, in part, to endogenous expression of the CDK6

protein. Our results also suggest that it might be possible to develop therapeutic agents, like I3C, that could be used to decrease CDK6 expression in patients with prostate cancer.

d. The roles of specific functional domains of the AR in mediating the stimulatory Effects of CDK6.

CDK activation of the AR pathway requires the full length AR. We found that deletions in the transactivation (ARΔ37-494) or the DNA binding (ARΔ557-653) domains markedly impaired the ability of CDK6 to stimulate PSA-luciferase reporter activity (Fig. 3). A mutant in the ligand binding domain (ARΔ653-910) which displays constitutive activity in the absence of DHT (10) was still active but not as active as the wildtype AR activity with respect to CDK6 stimulation of reporter activity (Fig. 3). Thus it appears that the ability of CDK6 to stimulate the activity of the AR requires multiple domains in the AR.

Increased length of trinucleotide repeats in the AR decreases CDK6 activation of the AR pathway. The transcriptional activation domain in exon 1 of the AR gene contains a polymorphic CAG repeat sequence (8). African American (AA) men with prostate cancer are more likely to have shorter CAG repeats in the AR than Caucasian men with prostate cancer. It has been suggested that this may explain, in part, the earlier age of onset of the disease and poorer prognosis in AA men (8). Therefore, it was of interest to determine whether CDK6 activation of the AR is dependent on CAG repeat length. Hence, we tested the effects of CDK6 activation on three AR constructs that vary in CAG repeat lengths, using the above described PSA- luciferase reporter assays. In the presence of DHT but absence of CDK6, AR proteins containing 48 (AR75), 20 (AR) or no (AR70) CAG repeats displayed approximately equal activities, although the protein with 48 repeats had somewhat lower activity. In the presence of DHT and CDK6, the AR protein with 48 repeats displayed negligible stimulation by CDK6, but the AR protein with no repeats gave an even higher stimulation by CDK6 than that obtained with the wildtype AR (20 CAG

repeats; Fig 4). These results indicate that stimulation of the AR by CDK6 is enhanced with ARs that contain shorter CAG repeats. Therefore, in AA men whose AR has short CAG repeats overexpression of CDK6 might exert an exaggerated stimulation of the AR and, thereby, markedly enhance the development of prostate cancer.

CDK6 activation of the AR pathway is markedly enhanced with the T877A mutant of the AR. The ligand binding domain of the AR is frequently mutated in human prostate cancer (25, 28). Mutations that convert leucine to histidine in codon 701 (AR L701H) and threonine to alanine in codon 877 (AR T877A) of the AR are of particular interest since these mutant ARs bind and are activated by steroids other than androgens. Thus, they may lead to the development of an androgen-independent state (9). Therefore, we examined the ability of CDK6 to enhance the transcriptional activities of these two mutant ARs in the above-described PSA promoter-luciferase reporter assays. In the presence of DHT, the activity of the mutant AR L701H was stimulated by CDK6 to about the same extent as that obtained with the wildtype AR (results not shown). However, in the presence of DHT, CDK6 produced a dramatic stimulation of the activity of the AR T877A mutant. Indeed, this activity was about 9-fold higher than the comparable activity obtained with the wildtype AR (Fig. 5). We then examined the effects of various steroid nuclear receptor ligands in similar PSA promoter reporter assays using cortisol, DHT, β-estradiol, flutamide and progesterone (Fig. 5). In the presence of AR T877A and absence of CDK6, β-estradiol and progesterone stimulated this reporter activity to an even greater extent than DHT. When wildtype AR was tested in the absence of CDK6, the other ligands did not stimulate reporter activity. When AR T877A was tested in the presence of CDK6, β-estradiol and progesterone caused a dramatic stimulation, which was equal to that obtained with DHT, and significant stimulation was also seen with cortisol and flutamide (Fig. 5). These results suggest

that in the subset of prostate tumors that express the T877A mutant form of the AR, CDK6 expression may play a critical role in enhancing the transition from an androgen-dependent prostate tumor to an androgen-independent tumor.

Key Research Accomplishments

1. We have confirmed and extended our discovery that the cell cycle control protein CDK6 can bind to and stimulate the transcriptional activity of the androgen receptor (AR) in human prostate cancer cells.

2. We have obtained further evidence that the above effect does not require the kinase activity of CDK6 and therefore CDK6 does not act by phosphorylating the AR.

3. The ability of CDK6 to stimulate the transcriptional activity of the AR is markedly affected by known naturally occurring variations in the amino acid sequence of the AR. African-American men who are at higher risk for prostate cancer then Caucasian or Asian men more frequently display shorter CAG repeats in exon 1 of the AR. We found that a decrease in CAG repeats in the AR enhances stimulation of its transcriptional activity by CDK6. This suggests that overexpression of CDK6 could be especially effective in stimulating the activity of the AR in African-American men, thereby enhancing the development of prostate cancer in these men.

4. A point mutated AR originally identified in human prostate cancers, designated AR T877A, displayed dramatic stimulation of transcriptional activity by CDK6 in the presence of DHT, β-estradiol or progesterone. These results suggest that in some cases of "androgen-independent"

prostate cancers, AR mediated pathways of gene expression that enhance growth are maintained, even when there are low levels of DHT or other steroids, by the stimulatory effects of CDK6 on mutant forms of the AR.

Reportable Outcomes:

During the past year a student in Dr. Weinstein's laboratory, J. Terry Lim, received his Ph.D. degree from Columbia University. This award played a critical role in supporting his thesis research. The results reported last year and this year constituted an important part of his Ph.D. thesis. An abstract describing these studies was submitted to the American Association for Cancer Research (AACR) and a related manuscript will be submitted for publication. This award and the results obtained also enhanced our ability to obtain funding for prostate cancer research from the T.J. Martell Foundation.

Conclusions:

This award has led to the novel discovery that the cell cycle control protein CDK6 can bind to the androgen receptor (AR) and stimulate its transcriptional activity in human prostate cancer cells. Furthermore, we found that this effect of CDK6 is markedly affected by naturally occurring variations in the amino acid sequence of the AR. These results may have important clinical implications with respect to why African American men with prostate cancer have a poorer prognosis. They may also help explain the mechanism by which prostate cancers often progress from an "androgen-dependent" to an "androgen-independent" stage. Finally, our results suggest that CDK6 may provide a prognostic marker in individual cases of prostate cancer and also a novel target for chemoprevention and therapy. Our proposed studies during the coming year should provide important additional information directly relevant to these potential clinical applications.

Future Studies:

Our current studies and studies to be completed during the coming year address Task 3 of our original Work Statement, namely to develop derivatives of LNCap cells that stably overexpress CDK6 and examine their phenotypic properties; including whether overexpression of CDK enhances the transcription of endogenous AR-responsive genes and whether it also enhances the growth of prostate cancer cells. These studies are crucial since it is important to demonstrate that the effects of CDK6 on the AR elucidated in Tasks 1 and 2 are not simply confined to reporter assays.

In our original Work Statement, the purpose of Task 4 was to develop transgenic mice that overexpress CDK6 in the prostate. However, two of the original reviewers questioned the wisdom of these studies because the mouse prostate is not an ideal model for studying prostate cancer. We agree with this criticism and also think that it is more important to directly examine the relevance of our findings to human prostate cancer. Therefore, we have initiated studies, using immunohistochemical methods, to determine whether there is increased expression of CDK6 in a cohort of human prostate cancer and whether or not this correlates with various clinical and pathologic parameters. Another goal for the coming year is to complete these studies and the data analysis. We are hopeful that these studies will indicate whether CDK6 may provide a useful prognostic marker and/or potential target for the prevention and treatment of prostate cancer.

Figure Legends

Fig. 1 Effects of mutations in CDK6 and the AR on the association between the two proteins. 239T cells were transfected with plasmids encoding HA- tagged wildtype (WT) or HA-tagged

point mutated forms of CDK6, with or without plasmid encoding WT AR. Lane 1, HA K6WT; lane 2, HA K6R31C; lane 3, HA K6D146N; lane 4, HA K6RE60, 61AA; lane 5, AR; lane 6, AR and HA K6WT; lane 7, AR and HA K6R31C; lane 8, AR and HA K6D146N; lane 9, AR and HA K6RE60, 61AA. The description of the HA plasmids indicates the sites of the amino acid changes. Whole cell extracts were immunoprecipitated with either the HA (top two panels) or the AR (bottom two panels) antibody and immunoblotted with either the HA or AR antibody.

Fig. 2 Dose-dependent inactivation of the effects of CDK6 by 13C. PC3 cells were transfected with the PSA luciferase reporter and the AR. The cells were then incubated with increasing concentrations of 13C for 24 or 48 hours, in the absence (□) or presence (■) of 10 nM DHT and relative luciferase activity was determined. Extracts of these cells were also immunoblotted for the AR and CDK6 as shown in the left panel.

Fig. 3 The effects of AR deletion mutants on CDK6 activation of the PSA promoter. PC3 cells were transfected with the PSA luciferase and β-gal plamids. As indicated, the cells were also co-transfected with one of the AR deletion constructs (AR Δ37-494, AR Δ653-910, AR Δ557-653 or the full length AR) and the full-length CDK6 expression plasmid (▥,■). Relative luciferase activity was then measured after the cells were grown in the absence (□,▥) or presence (■,■) of 10nM DHT for 20 hours. The structures of the deletion mutants are shown schematically on the left.

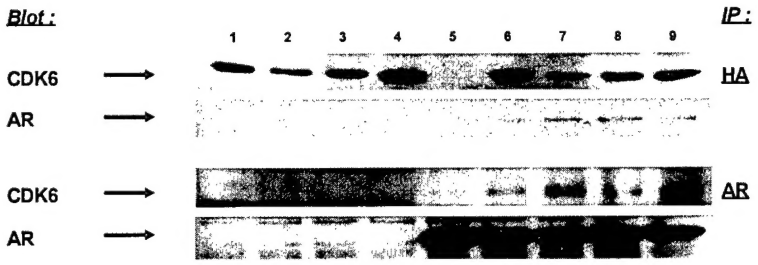
Fig. 4 The effects of CAG polymorphism in the AR on CDK6 activation of the PSA promoter. PC3 cells were transfected with the PSA luciferase reporter and β-gal plamids. As indicated, the cells were also co-transfected with an AR plasmid encoding 48 (AR75), 20 (AR) or no (AR 70) CAG repeats and CDK6.

Fig. 5 The effects of an AR mutant found in human prostate cancer on CDK6 activation of the PSA promoter in the presence of various steroids. PC3 cells were transfected with the PSA luciferase reporter and β-gal plamids. As indicated, the cells were also co-transfected with wildtype AR or the AR mutant AR T877A and CDK6 and grown in the absence or presence of cortisol, DHT, β-estradiol, flutamide or progesterone. *, $P < 0.05$.

References:

1. S. H. Landis, T. Murray, S. Bolden, P. A. Wingo, *CA Cancer J Clin* **49**, 8-31, 1 (1999).
2. P. Boyle, P. Maisonneuve, P. Napalkov, *Eur Urol* **29**, 3-9 (1996).
3. A. Sgambato, G. Flamini, A. Cittadini, I. B. Weinstein, *Tumori* **84**, 421-33 (1998).
4. J. Zhang, T. Z. Thomas, S. Kasper, R. J. Matusik, *Endocrinology* **141**, 4698-710 (2000).
5. C. Albanese, et al., *Mol Biol Cell* **14**, 585-99 (2003).
6. M. J. Grossel, G. L. Baker, P. W. Hinds, *J Biol Chem* **274**, 29960-7 (1999).
7. E.J. Cran,et al., *J. Biol Chem* **276,** 22332-40 (2001).
8. C.S. Choong et al., *J. Mol Endocrinology* **21,**235-57 (1998).
9. X.Y. Zhao,et al., *Nat Med* **6,** 703-6 (2000).
10. A.O. Brinkman, et al., *J. Steroid Biochem Mol Bio* **69,** 307-13 (1999)

Fig. 1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3
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9
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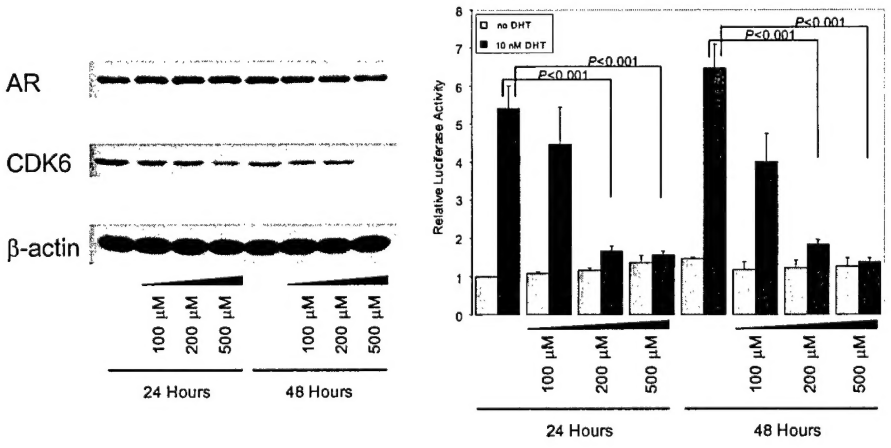
HA
CDK6
AR
AR

Fig. 2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500 µM
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200 µM
500 µM
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48 Hours
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P<0.001
P<0.001
P<0.001
Relative Lucferase Activity
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1
2
3
4
5
6
7
8
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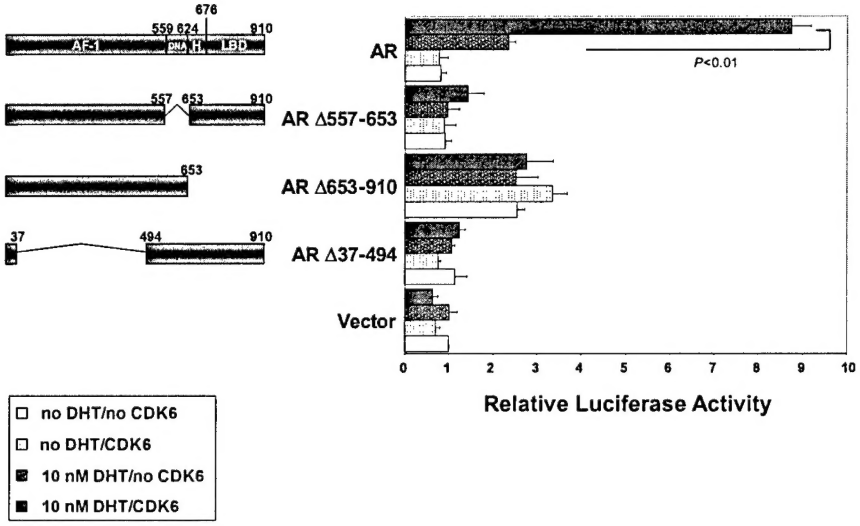
200 µM
500 µM
100 µM
200 µM
500 µM
24 Hours
48 Hours

Fig. 3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910
AF-1
NH
H
LBD
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910
653
37
494
910
AR
AR Δ557-653
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AR Δ37-494
Vector
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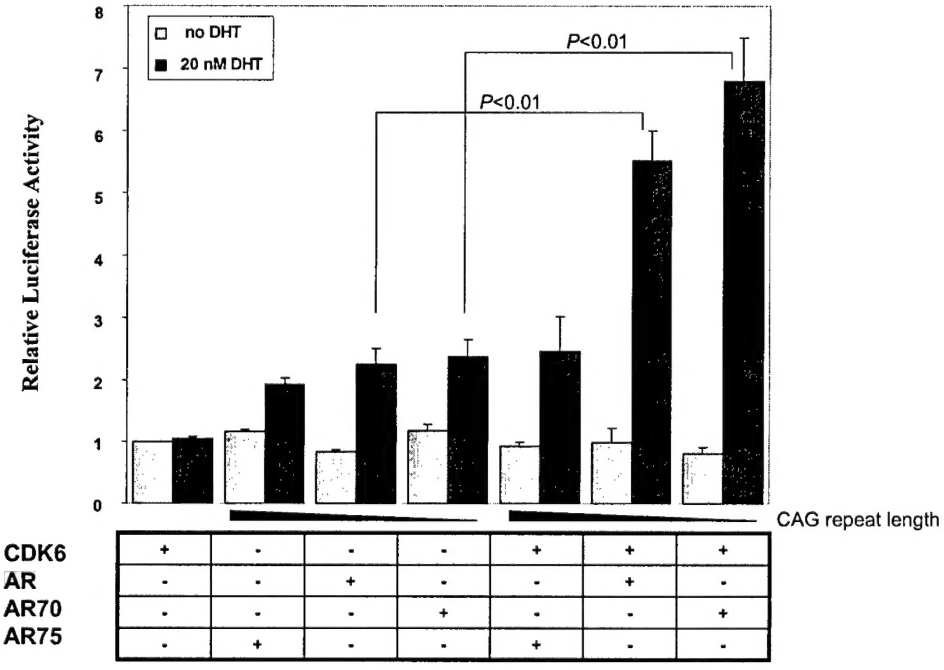
10 nM DHT/CDK6

Fig. 4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P<0.01
Relative Luciferase Activity
0 1 2 3 4 5 6 7 8
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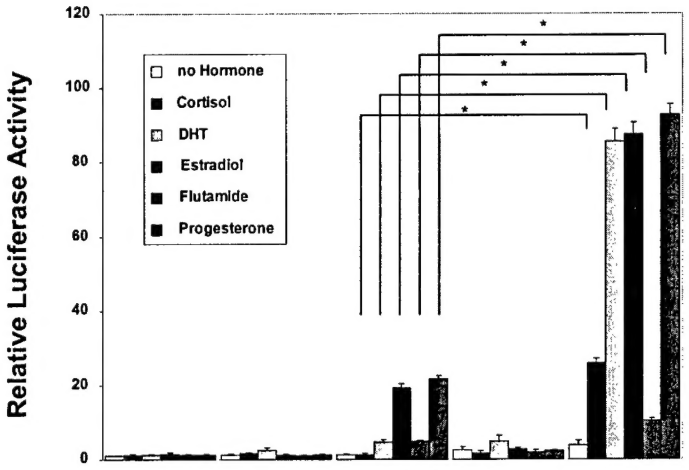
AR - - + - - + -
AR70 - - - + - - +
AR75 - + - - + - -

Fig. 5



CDK6	+	-	-	+	+
AR	-	+	-	+	-
AR T877A	-	-	+	-	+